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PRINCIPAL INVESTIGATOR: Dr. Gregory Foltz

CONTRACTING ORGANIZATION: Swedish Health Services

Seattle, WA 98122-4379

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Dr. Gregory Foltz

E-Mail: greg.foltz@swedish.org

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14. ABSTRACT

This research proposal will provide important insights into cancer mechanisms and blood biomarkers to assess progression and stratification of human glioblastoma. We have collected several tumor specimens to date and have made excellent progress in establishing primary tumor cell cultures from individual tissue samples. These parental cell lines have successfully been used for the generation of quantized tumor cell populations for general molecular characterization, and for evaluating the effectiveness of drug candidates in targeting these cell populations. This program will significantly advance genomic, proteomic and single-cell technologies, which will be generally applicable to all cancer-based studies.

15. SUBJECT TERMS

Human cohorts, Glioblastoma, Genomic, Proteomic, Single-cell technologies, Hypothesis-driven, integrative systems approach, Early diagnosis, Patient stratification, Blood protein biomarkers, Quantized cell populations

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Table of Contents

INTRODUCTION

This collaborative research program will provide important insights into human cancer mechanisms. In particular, we intend to develop quantitative tools with direct applications for patients with glioblastoma multiforme (GBM), the most common and most aggressive brain tumor in adults. Despite treatment strategies that include gross total resection, post-operative radiation and chemotherapy, most patients develop tumor recurrence within months. Cancer stem cells have been implicated as the presumed cause of tumor recurrence and resistance to therapy. With this in mind, we will utilize GBM patient-derived cell lines and new technologies for transcriptome, miRNAome, proteome, and single-cell analyses to study quantized GBM cell populations and their role in disease progression. This proposal will significantly advance genomic, proteomic and single-cell technologies. The tools proposed here will be generally applicable to all cancer-based studies, as the nature of the tool development is designed to identify and quantify DNA, RNAs, proteins and cells, challenges ubiquitous to all human disease systems.

The expected outcomes of this research program will include: 1) a deeper understanding of human GBM disease mechanisms; 2) blood protein biomarkers for use in early diagnosis, assessment of GBM progression, evaluation of drug treatment effectiveness, and early detection of disease recurrence; 3) new strategies for advanced genomic sequencing of quantized cancer cells and their normal counterparts to identify cancer-driver mutations; 4) new technologies for transcriptome, miRNAome, proteome, and single-cell analyses, and 5) the creation of quantized GBM cell lines that can be used for general molecular characterization as well as to assess GBM biology and the effectiveness of existing drugs in reacting with these cell types. To achieve these goals we are pursuing the following aims:

Specific Aim 1. Isolate up to 1000 cells from each of five human GBMs and quantify initially 500 different transcripts from each cell (transcription factors, CD molecules, relevant signal transduction pathways, etc). Determine whether computational analyses can classify these cells into discrete quantized cell types.

Specific Aim 2. Sort the disassociated tumor cells from GBM into their quantized cell populations using cell sorting/CD antibodies to each quantized cell type for functional analyses and establish primary cell lines. These will be used for molecular analyses at the genome, transcriptome, miRNAome and selected proteome levels.

Specific Aim 3. Assess 20-40 candidate blood biomarkers in the bloods of 100 GBM patients with regard to their ability to stratify disease, assess disease progression and predict at an early stage GBM recurrence. Eventually we will use these biomarkers to assess the effectiveness of therapy.

Specific Aim 4. Ten to 20 cells from each major quantized GBM cell type from two patients will be used to determine the complete genome sequences. We will also determine the normal genome sequences of each patient and their family members to enable the Mendelian-based error correction process (*1*). The mutations will be analyzed against quantitative changes in the transcriptomes, miRNAomes and proteomes and against the relevant biological networks.

Specific Aim 5. Analyze the quantized cell populations for their responses (transcriptome, miRNAome, etc) to the perturbations of key GBM-relevant molecules (e.g. nodal points in networks) by RNAi perturbations as well as their responses to GBM-relevant drugs and natural ligands.

BODY

This project was initially set to begin June 15, 2011, however delays in IRB approval prevented us from starting at the appointed date. We received IRB approval in April of 2012 and were able to start work in July 2012. We therefore submitted a request on June 14, 2013 for a one time no cost extension for the project period to be extended to June 14, 2014 to provide us with sufficient time to complete the proposed studies. As requested, we have supplied SF425 for December 2012 and March 2013. We hope to be issued an award modification extending the period of performance soon.

The Ivy Center for Advanced Brain Tumor Treatment at the Swedish Neuroscience Institute (SNI) has collected tumor tissue eligible for this program from over forty GBM patients to date. We have made excellent progress in the establishment of primary GBM cell lines from patients undergoing tumor resection at SNI. In brief, tumor samples are treated with Accutase (Sigma) immediately after surgical resection, and single cell suspensions are plated in NeuroCult® NS-A media with epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) as described (*2*). Although the criteria that define cancer stem cells are controversial (*3*-*6*), it is generally accepted that GBM stem cells must demonstrate: (1) self-renewal (*i.e.* serial neurosphere formation from a single cell), (2) multipotency (*i.e.* the ability to differentiate into cells expressing neuronal and glial cell markers), and (3) tumor-initiating ability *in vivo* (*7*). Our patient derived cultures contain cells capable of forming self-renewing spheres, signifying the presence of a self-renewing stem cell population. Immunocytochemistry (ICC) studies have verified the expression of common neural stem cell markers (Figure 1A-C), and the ability of cells to differentiate into astrocytes, neurons and oligodendrocytes (Figure 2A-C).

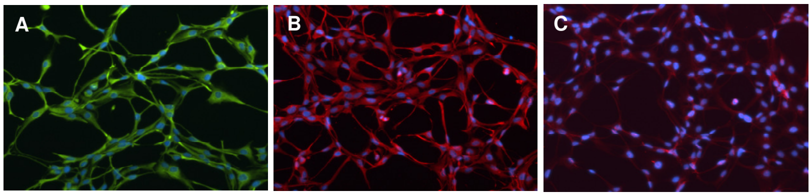

Figure 1. Immunostaining for neural stem cell markers: (A) nestin, (B) vimentin and (C) CD44. Primary antibodies were from R&D Systems and goat secondary antibodies conjugated to Alexa dyes were from Invitrogen. DAPI (Sigma) was used as the nuclear counterstain. Images were acquired using a Nikon Ti-U inverted fluorescence microscope liked to a DS-U2 camera.

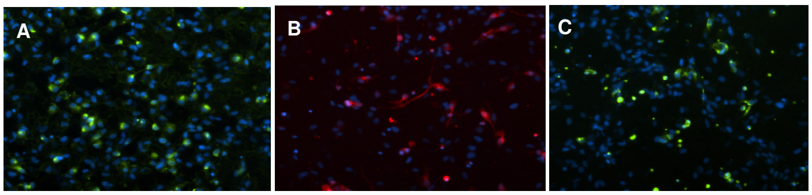

Figure 2. Immunostaining for differentiation markers: (A) GFAP/astrocytes, (B), TUJ-1/neurons and (C) O4/oligodendrocytes. Cells were grown in NSA media without growth factors (EGF and FGF-2) for 2 weeks. Other experimental conditions are as in Figure 1.

We have evaluated tumorigenicity *in vivo*, and observe tumor formation in NOD/SCID mice approximately 6-8 weeks after cell inoculation (Figure 3). Furthermore, we have confirmed with a board certified neuropathologist that the harvested xenograft tumor tissue is consistent with GBM (Figure 4). These results collectively demonstrate the presence of stem-like cells within our patient-derived GBM cultures, and verify that these primary cell lines are suitable for the proposed research.

We have transferred several GBM-derived cultures from SNI to our collaborators at the Institute for Systems Biology (ISB; Award Number W81XWH-11-1-0487, Dr. Robert Moritz) for the generation of quantized cell populations for molecular analyses at the genome, transcriptome, miRNAome and proteome levels (Specific Aims 1 and 2). To determine whether there are quantized cell populations within primary GBM cells, we proposed transcriptomic profiling of hundreds of single cells from five patients. Expression patterns of the selected genes among these single tumor cells would digitally stratify tumor cells into distinct populations. Single cell gene expression analysis, using the Fluidigm Biomark and NanoString nCounter platforms, has been successfully performed on two patient samples (SN143 and SN186) to date. By analyzing over 100 single cells using a panel of more than 20 markers, at least three distinct cell populations are observed (see PI Moritz report). These results demonstrate that single cell gene expression assays are capable of identifying intratumoral cellular heterogeneity and moreover, that there are quantized cell populations in primary GBM cells. The target gene panel (up to 500 genes to include key cancer signaling molecules, transcription factors, in addition to the cell surface CD markers), will be analyzed in additional patient samples (n=5) as proposed.

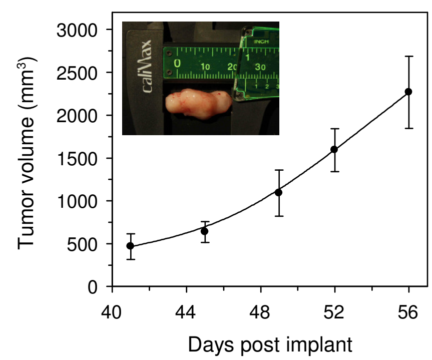

Figure 3. Average tumor volume (n = 5) in immunocompromised mice six weeks after implantation of GBM-patient derived cells (SN186). *Inset*: Tumor mass removed from host. Tumor initiation *in vivo* confirms the presence of stem cell populations within the heterogeneous cell culture.

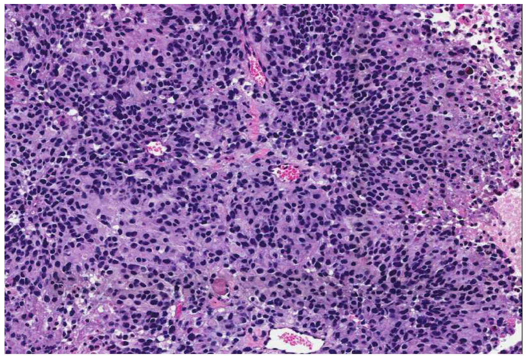

Figure 4. Histology (H&E staining) of xenograft tumor tissue derived from primary GBM cell lines. Staining results are consistent with “classic” GBM with angiogenesis, pseudopalisade pattern and areas of necrosis.

A number of quantized cell populations have been established from the SN143 and SN186 parental cell lines by single cell sorting using the BD FACS Aria II. Single cells are seeded into 384-well plates for the expansion of clonal cultures. The quantized cell populations exhibit distinct morphological phenotypes and different colony-forming capabilities. Each clone presumably carries a uniform genome, and is therefore ideal for whole genome sequencing. To date, whole transcriptomics analysis has been performed on selected clones from both patient samples in order to evaluate molecular heterogeneity at the transcript level. The observed cell population distribution pattern is consistent with the single cell gene expression analysis. From these combined analyses, a panel of 48 genes that potentially function as GBM subpopulation-specific markers has been established. This panel is being evaluated for SRM-based targeted proteomics assays (see PI Moritz report).

For biomarker assessment by proteomics analysis, we are actively collecting blood samples from GBM patients to allow the isolation of plasma as well as peripheral blood mononuclear cells (PBMCs). A significant challenge is to preserve proteomic sample integrity for accurate analyses, as proteolysis occurs within minutes of blood collection resulting in the rapid degradation of plasma proteins and peptides. We have a standardized method in place which prevents the loss of proteins and peptides whilst yielding high-quality hemolysis-free plasma (Figure 5). The identification of proteomic biomarkers in human plasma holds incredible clinical potential. The plasma collected from GBM patients will be used to assess candidate blood biomarkers useful in early diagnosis, stratification, assessment of GBM progression, and early detection of disease recurrence (Specific Aim 3). Methodology for selected reaction monitoring (SRM) assays has been established, and will be used to detect and quantitate candidate proteins as GBM signature markers.

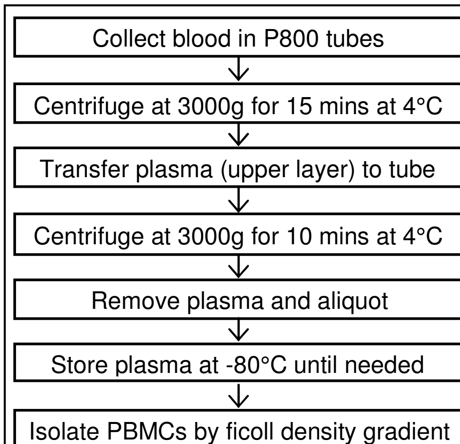

Figure 5. Established protocol for the isolation of human plasma and PBMCs from whole blood samples.

Cells into 384-well plates (1000/well)

↓

Cells attach overnight (37°C, 5% CO_2)

↓

Add different compounds (0.01-10 μM)

↓

Incubate for 96 hrs (37°C, 5% CO_2)

↓

Add Cell Titer-Glo for cell lysis (15 mins)

↓

Record luminescent signal on Envision

↓

Signal = ATP = metabolically active cells

Figure 6. Protocol for high throughput chemical screens. Viable cells (metabolically active) are determined by ATP quantification, using the CellTiter-Glo luminescent cell viability assay (Promega).

For whole genome sequencing, blood cells from patients and their family members are required (Specific Aim 4). As expected, recruiting both patients and family members is a difficult task. However, we have been able to collect blood samples from one GBM patient (SN291) and three of the patient's family members. We have identified several other eligible patients and hope to consent additional families shortly. We will collect blood from these individuals for complete genomic sequencing according to our initial quantized single-cell analyses. Our collaborators at ISB have established the genomics pipeline necessary for the Mendelian-based analyses of family genome data in addition to the more routine analyses for cancer-relevant mutations (*1*).

We have established high-throughput screening (HTS) technology for testing multiple compounds against GBM-derived cell lines (Figure 6). In addition, we have optimized HTS assays for performing dose response studies, used to evaluate drug potency. Dose response curves are used to determine the half maximal inhibitory concentration (IC_{50}), which represents the compound concentration required for 50% tumor cell inhibition *in vitro*. Preliminary results obtained with one drug are presented in Figure 7. This methodology will be applied to the quantized GBM cell populations to analyze their responses to multiple chemotherapy agents (Specific Aim 5).

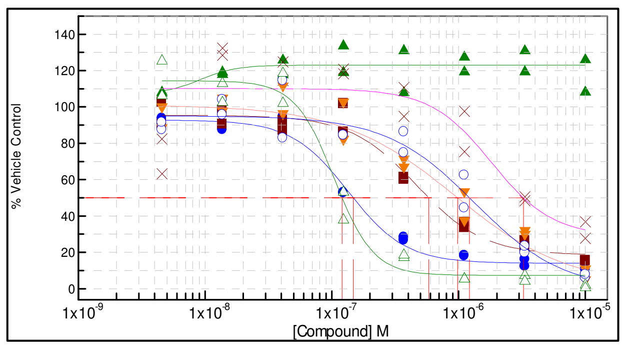

Figure 7. Dose response curves for multiple primary GBM cell lines. Normal human NSCs (control) are represented by solid green triangles. Assays were performed as described in Figure 6. IC_{50} values were calculated by fitting dose response curves to the four-parameter nonlinear regression model.

KEY RESEARCH ACCOMPLISHMENTS

In summary, the following have been established for the research program of this grant:

- **Tumor collection:** The Ivy Center for Advanced Brain Tumor Treatment at the Swedish Neuroscience Institute (SNI) has collected potentially eligible tumor tissue from over forty GBM patients.

- **Primary GBM cell lines:** we have refined tissue processing techniques to allow for the

routine establishment of GBM patient-derived primary cell lines that preserve the stem cell phenotype.

- **Quantized cell populations:** single cell gene expression assays have identified quantized cell populations in primary GBM cells. Several quantized cell populations have been established (from SN143 and SN186 cell lines) and expanded for further studies.

- **Methodologies for whole genome sequencing, transcriptomics, and proteomics analyses:** have been applied to quantized cell populations from SN143 and SN186 to yield promising data. A panel of genes that potentially function as GBM subpopulation-specific markers has been established for SRM-based targeted proteomics assays.

- **Blood biomarker studies:** we are actively collecting blood samples from GBM patients to assess candidate blood biomarkers using SRM assays in a large patient cohort.

- **Family sequencing:** we have consented one family for whole genome sequencing analysis and blood plasma collection for the downstream proteomic analysis of defined GBM targets. We have processed blood from the patient (SN291), one sibling and two children.

- **High-throughput chemical screening:** we have optimized high-throughput screening methodology using the parental GBM cell lines for informer screens and subsequent dose response studies. Similar assays will be used to profile drug responses of the quantized cell populations.

REPORTABLE OUTCOMES

No reportable outcomes have been established for the 2012/2013 period.

CONCLUSION

Description of work to be performed during the next reporting period:

We will complete the 5 patient cohort, isolate up to 1000 cells from each GBM and quantify 500 different transcripts from each cell (Aim 1). The quantized cell populations established from SN143 and SN186 are not amenable for subsequent whole genome sequencing and plasma proteomics analysis because the patient is deceased, and no peripheral blood mononuclear cells (PBMCs) or plasma are available. To address this, we identified SN291 for which PBMCs and plasma are readily available. The SN291 primary cell line has been

transferred to ISB for the generation of quantized cell populations. It is important to note, that work with the previous quantized cells has enabled us to refine techniques for studying future samples (Aims 1 and 2). Our focus now will be establishing clonal cultures from SN291 whose family members have also consented to family genome sequencing and blood collection. We anticipate in the coming few months we will be able to generate enough materials from this patient family for whole genome sequencing and proteomics analysis.

We will complete our selection of 64 patients for proteomic analysis, maximally leveraging data generated from the TCGA and Allen atlas projects. Promising candidate target proteins will be validated on all 64 patient tumor and blood samples using targeted proteomic SRM approaches. We will continue to collect blood and isolate plasma from additional GBM patients.

We will consent additional families for whole genome sequencing analysis and blood plasma collection. The corresponding patient-derived cell line will be transferred to ISB for the generation of quantized cell populations to determine complete genome sequences. The normal genome sequences of each patient and their family members will also be determined. Cancer-relevant mutations will be analyzed against quantitative changes in the transcriptomes, miRNAomes and proteomes and against the relevant biological networks.

We will also focus on measuring the drug potency of commonly used chemotherapy agents against parental GBM cell lines. The IC_{50} for parental cell lines versus the IC_{50} for quantized cell populations will provide a direct measure of drug responses (Specific Aim 5). We hope this will provide valuable information that can be translated to the clinic and used to design effective treatment strategies for future patients.

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APPENDICES

N/A